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AR and ER Interaction with a p21-Activated Kinase (PAK6)

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A human protein termed p21-activated kinase 6 (PAK6), based on homology to the PAK family of serine/threonine kinases, was cloned as an AR interacting protein. PAK6 was a 75-kDa protein with a predicted N-terminal Cdc42/Rac interactive binding domain and a C-terminal kinase domain. PAK6 bound strongly to GTP-Cdc42 and weakly to GTP-Rac. In contrast to most PAKs, kinase activity was not stimulated by Cdc42 or Rac, but could be stimulated by AR binding. PAK6 interacted with the intact AR in a mammalian one-hybrid assay and bound *in vitro*, without ligand, to the hinge region between the AR DNA- and ligand-binding domains. PAK6 also bound to the ERα, and binding was enhanced by 4-hydroxytamoxifen. AR and ERα transcriptional activities were inhibited by PAK6 in transient transfections with episomal and integrated reporter genes. AR inhibition was not reversed by transfection with an activated Cdc42 mutant, Cdc42V12, which by itself also inhibited AR transactivation. Epitope-tagged PAK6 was primarily cytoplasmic in the absence or presence of AR and hormone. PAK6 transcripts were expressed most highly in brain and testis, with lower levels in multiple tissues including prostate and breast. PAK6 interaction provides a mechanism for cross-talk between steroid hormone receptors and Cdc42-mediated signal transduction pathways and could contribute to the effects of tamoxifen in breast cancer and in other tissues. (*Molecular Endocrinology* 16: 85–99, 2002)

THE AR is a steroid hormone receptor member of the larger nuclear receptor family that mediates the biological functions of androgens (1, 2). In addition to its physiological roles in many tissues, the AR also plays a central role in prostate cancer development and progression (3, 4). The steroid hormone receptors share a relatively conserved central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD), which also has a ligand-dependent transactivation function (referred to as AF-2). Their N-termini are more diverse, but generally possess an independent transactivation function (AF-1), with the AR N-terminal domain being particularly large and having a strong AF-1. Hormone binding causes conformational changes that result in receptor dissociation from an HSP90 chaperone complex, homodimerization, and the generation of a binding site for proteins containing leucine-x-x-leucine-leucine (LXXLL) motifs, including the p160 family of steroid receptor coactivator (SRC) proteins (5–7). The SRC proteins appear to be the major coactivators mediating the ligand-dependent AF-2 transactivation function of the LBD, through histone acetyl or methyltransferase activity and association with cAMP response element binding protein or p300. The SRC proteins can also interact directly with the N terminus of steroid hormone receptors, and this interaction may be particularly critical for the AR (8–11).

In addition to the p160 steroid receptor coactivator proteins, there is a growing list of proteins that interact with the N-terminal, DNA, or ligand binding domains of steroid hormone receptors (5, 6). These include proteins linked to the general transcriptional machinery, proteins that function as transcriptional coactivators or corepressors by other mechanisms, and proteins the functions of which remain to be determined. Some of these interactions are regulated by ligand binding and mediated by LXXLL or related motifs, while others are independent of ligand. There is also a ligand- dependent interaction between N-terminal and C-terminal domains of the AR and other steroid hormone receptors, which appears to be particularly important for AR transactivation (12–18). Finally, there is evidence for nontranscriptional functions of steroid hormone receptors (19–21) and an association between the ERα and PI3K (22).

Abbreviations: ARE, Androgen response element; BIC, bicalutamide; CDS, charcoal dextran-stripped; CRIB, Cdc 42/Rac interactive binding; DBD, DNA-binding domain; DHT, dihydrotestosterone; DTT, dithiothreitol; ERE, estrogen response element; EST, expressed sequence tag; GFP, green fluorescent protein; GRIP, GR-interacting protein; GST, glutathione-*S*-transferase; LBD, ligand-binding domain; mAb, monoclonal antibody; MBP, myelin basic protein; MMTV-LTR, mouse mammary tumor virus-long terminal repeat; NLS, nuclear localization signal; OHT, 4-hydroxytamoxifen; PAK, p21-activated kinase; SRC, steroid receptor coactivator.

This report describes the isolation and characterization of an AR and ERα interacting protein termed p21-activated kinase 6 (PAK6), based upon its homology to previously identified PAKs. PAKs form an evolutionarily conserved family of serine/threonine kinases that bind to, and are regulated by, the active (GTP-bound) form of the Rho family small (p21) GTPases, Cdc42 and Rac (23–26). Cdc42 and Rac binding are mediated by a conserved N-terminal Cdc42/Rac interactive binding (CRIB) domain (27). PAKs are presumed to mediate some of the downstream effects of activated Cdc42 and Rac, although the targets of their kinase activity and precise functions remain to be determined. The yeast PAK homolog (*STE20*) activates a MAPK kinase kinase analogous to mammalian Raf (28), and mammalian PAKs have been reported to similarly activate MAPK pathways in response to activated Cdc42 and/or Rac (29–34). Additional possible roles for PAKs are in cytoskeleton organization (35–37), cell cycle regulation (38), heterotrimeric G protein signaling (39), and apoptosis (40–43). Therefore, the AR- and ERα-PAK6 interactions provide potential direct links between these steroid hormone receptors and signal transduction pathways regulating diverse cellular functions.

RESULTS

Isolation of a PAK-Related Kinase Interacting with the AR in Yeast

A fragment of the human AR containing the DNA and ligand binding domains (AR505–919), was fused to the GAL4 DNA binding domain and used as the bait in a series of yeast two-hybrid screens, in the presence of 1 μM dihydrotestosterone (DHT). Positive clones were subsequently screened without DHT, and several clones that showed strong DHT-dependent growth were isolated and sequenced. Two clones contained in-frame fusions to previously identified proteins, gelsolin, an actin-binding protein (44), and ARA70. ARA70 (fused at alanine 168) was identified previously as an AR interacting protein, although its functional significance remains unclear (45, 46). The significance of the AR interaction with gelsolin was also unclear, although an AR interaction with another actin-binding protein, filamin, was recently reported (47).

Partial sequencing of a third isolate (clone 56) indicated that it was a novel protein. A specific interaction between the AR and clone 56 in yeast was confirmed by cotransforming clone 56 (fused to the GAL4 transactivation domain) with additional plasmids encoding GAL4 DNA-binding domain (GAL4 DBD) fusion proteins and assessing β-galactosidase production from an integrated GAL4 responsive reporter. In the absence of DHT, only low levels of β-galactosidase activity were detected in all cases (not shown). DHT increased β-galactosidase activity 27-fold in yeast expressing both clone 56 and the GAL4 DBD-AR (505–919) fusion protein used in the yeast screen (Table 1). This level of induction was greater than that seen with a transactivation domain fused to GR interacting protein 1 (GRIP1) (563–1121), which contains three LXXLL motifs and an additional AR-interacting domain (48). In contrast, no induction was observed when clone 56 was expressed with GAL4 DBD fusion proteins containing the AR N-terminal transactivation domain, AR (2–506), the DBD and nuclear localization signal (NLS), AR (553–635), or with the irrelevant protein cortactin. These results demonstrated that the protein encoded by clone 56 interacted with the AR in yeast and that the interaction required a region C-terminal to the NLS.

Sequence Analysis of Full-Length PAK6

Complete sequencing of clone 56 revealed a consensus kinase domain at the C terminus, but no homology to previously reported proteins at the amino terminus (Fig. 1A). However, the first 35 nucleotides of the cDNA insert (nucleotides 874–908, *boxed* in Fig. 1A) were identical to the 3′-end of an expressed sequence tag (EST) from a testis cDNA library (GenBank accession no. AA815255), indicating that this EST encoded the 5′-end of clone 56. The plasmid containing this EST was obtained and sequenced to provide the 5′-end of the transcript. The assignment of the initiation methionine was based upon an in-frame stop codon (tga) 39 bases upstream (Fig. 1A, *boxed*). Since the overlap between the EST and clone 56 was only 35 bases immediately before a poly A tract in the EST, RT-PCR was used to confirm that this EST represented the 5′-end of the clone 56 transcript. RT-PCR from prostate cancer-derived cDNAs using 5′-primers derived from the EST and 3′-primers from clone 56 generated a product of the predicted size and sequence (data not shown), confirming that EST AA815255 represented the 5′-end of clone 56.

The full-length sequence predicted a protein of 681 amino acids with a molecular mass of 75 kDa (Fig. 1A). Analysis of the full-length coding region revealed homology at the 5′- and 3′-ends to the PAK family of serine/threonine kinases, of which four had been previously reported in humans (24, 37, 49–51). The sequences of two additional human PAK-related pro-

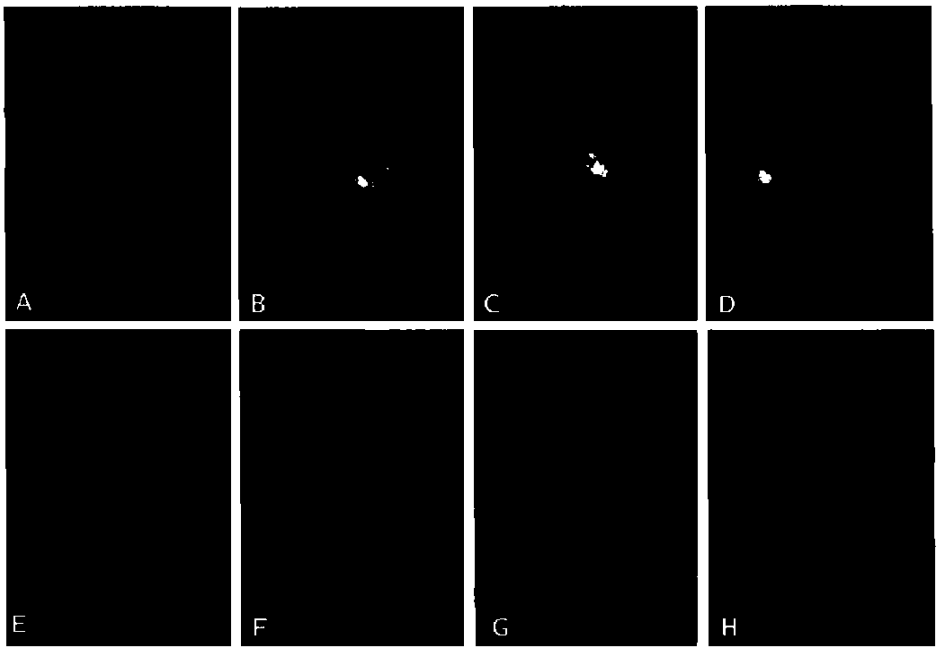
Table 1. AR Interaction with Clone 56 in Yeast

Bait	Prey	Fold Induction
pAS2-AR(2–506)	pACT2 clone 56	1.1
pAS2-AR(553–635)	pACT2 clone 56	0.9
pAS2-AR(505–919)	pACT2 clone 56	27
pAS2 vector	pACT2 clone 56	1.2
Cortactin	pACT2 clone 56	1.0
pAS2-AR(505–919)	GRIP1(563–1121)	5.7

Fold induction is based on β-galactosidase activity minus *vs.* plus 1 μM DHT. GRIP1 was in pGAD24.

Fig. 1. Complete Sequence of PAK6 and Homology to Other PAKs

A, PAK6 nucleotide and predicted amino acid sequence. An in-frame stop codon (tga) 5′ of the predicted initiation ATG and the N-terminal CRIB domain are *boxed*. Nucleotides at the 5′-end of yeast clone 56 that were identical to the 3′-end of the EST from a testis library (nucleotides 874–908) are also *boxed*. Amino acids highly conserved in all kinase domains are *boxed*. S531, which is an asparagine in most other kinases, is *boxed* and *in bold*. A possible heterotrimeric G protein-binding site is *boxed* at the C terminus. B, Homology in CRIB and autoinhibitory domains. The alignments are based on PAK6 with *periods* (.) indicating identical residues and *dashes* (–) marking gaps introduced to maximize homology. PAK1–3 have an N-terminal extension past the CRIB domain that is absent in PAK4–6. β-Strands (β1 and β2) and α-helices (H1–3) are from the PAK1 crystal structure. Residues *underlined* in the CRIB domain mediate dimerization. The autoinhibitory lysine in PAK1–3 is indicated by an *asterisk* (*).



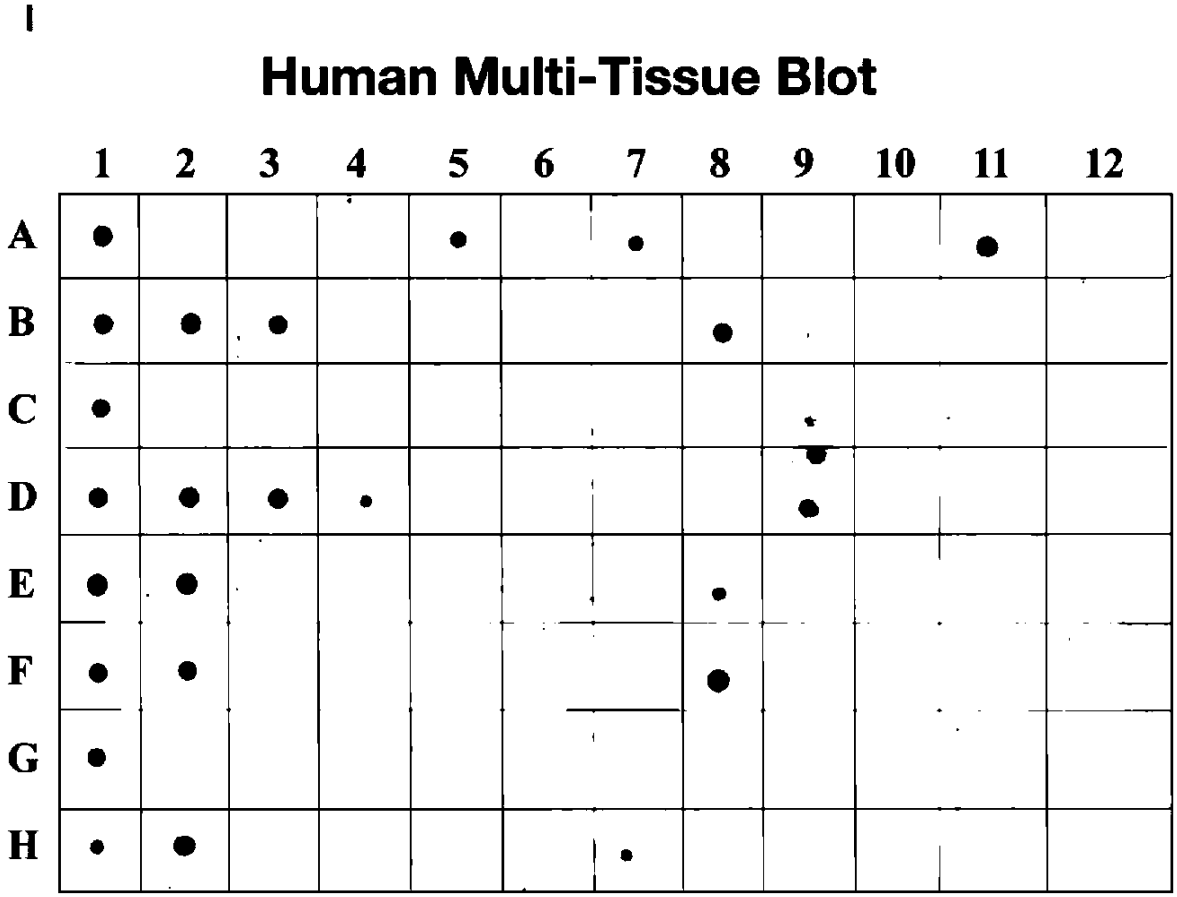


Fig. 2. PAK6 Cell and Tissue Distribution

A–D, HeLa cells transiently transfected with GFP-PAK6 (pEGFP-PAK6) alone (A and C) or cotransfected with the pSVAR$_o$ expression vector (B and D). E–H, HeLa cells transiently transfected with GFP-AR (pEGFP-AR) alone (E and G) or cotransfected with pcDNA-PAK6 (F and H). Cells were in steroid hormone-depleted medium (A, B, E, and F) or were treated for 30 min with 10 nM DHT (C, D, G, and H). I, Multiple tissue expression array probed with unique ^{32}P-labeled fragment of PAK6. Column 1 (A–H),

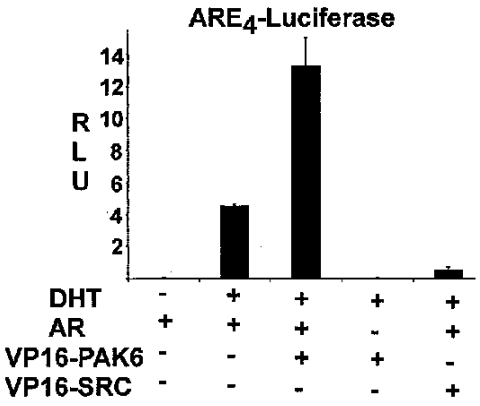


Fig. 3. Mammalian One-Hybrid Analysis of PAK6-AR Binding

CV1 cells were cotransfected with ARE_4-Luc reporter (100 ng), pRL-SV40 (Renilla control), and with the $pSVAR_o$ (200 ng), VP16-PAK6(256–681) (20 ng), or VP16-SRC(595–780) (20 ng), as indicated. DHT was added at 24 h, and cells were harvested at 48 h and assayed for luciferase and Renilla activity.

teins, termed PAK5 and PAK6, were more recently deposited in GenBank. The protein isolated here was identical to PAK6, located on chromosome 15q15.

The N terminus of PAK6 had homology with the CRIB domains of the previously characterized PAKs, containing six of eight of the CRIB domain consensus residues (Fig. 1B) (27). The greatest homology was with PAK4 (37) and PAK5, also with CRIB domains at the immediate N terminus. The recently reported crystal structure of human PAK1 indicated that this protein formed a dimer through an antiparallel β-ribbon formed by a β-strand that overlapped the CRIB domain (β1 in Fig. 1B) (52). The critical contact residues in this β-strand are conserved in PAK1–6 (*underlined* in the CRIB consensus in Fig. 1B), suggesting that PAK6 is similarly a dimer. The PAK1 crystal structure also showed that the autoinhibition of PAK1 kinase activity was due to a bundle of three helices (H1–3 in Fig. 1B) that packed against the kinase domain and positioned a lysine residue (amino acid 141 in PAK1, indicated with an *asterisk*) into the active site. These structural features were highly conserved in PAK1–3, but were not evident in PAK4–6, suggesting alternative mechanisms for regulating the kinase activity of these latter PAKs.

The C terminus of PAK6 encoded a consensus kinase domain with homology to PAK1, 2, and 3 (50% compared with PAK1), but again with much greater homology to PAK4 (80% homology). Residues highly conserved in kinase domains were also conserved in PAK6 (Fig. 1A, *boxed*), with the exception of an asparagine that was replaced by a serine (S531), a replacement also seen in PAK4 (*boxed and in bold* in Fig. 1A). Other conserved structural features of previously described PAK family members include N-terminal proline-rich SH3-binding motifs, which can target PAKs to the membrane through the Nck adapter protein (31, 53–55), and a heterotrimeric G protein β-subunit binding domain at the C terminus (39, 56). The former N-terminal SH3-binding motifs were not present in PAK4, 5, or 6, as the CRIB domains were at the extreme N terminus. Three of the four residues defining a putative heterotrimeric G protein binding motif were present in the C terminus of PAK6 (Fig. 1A, *boxed*).

Cell and Tissue Distribution of PAK6

The full-length PAK6 was fused to the C-terminus of green fluorescent protein (GFP) and used to assess cellular distribution. The GFP-PAK6 fusion protein transfected into HeLa cells localized primarily to the plasma membrane and cytoplasm (Fig. 2A). PAK6 remained primarily cytoplasmic when cotransfected with the AR, in the absence (Fig. 2B) or presence of DHT (Fig. 2D). HeLa cells were similarly transfected with a GFP-AR expression vector to assess PAK6 effects on AR distribution. The AR in HeLa cells was primarily cytoplasmic in the absence of DHT (Fig. 2, E and F) and nuclear in the presence of DHT (Fig. 2, G and H). This distribution was not altered by PAK6 cotransfection (Fig. 2, F and H). Similar results were obtained by indirect immunofluorescence with AR and an epitope-tagged PAK6, indicating that cellular localization was not altered by the GFP fusion (data not shown).

Hybridization of a unique internal fragment from PAK6 (nucleotides 451-1256) to a human multiple tissue expression array revealed the strongest expres-

Whole brain, cerebral cortex, frontal lobe, parietal lobe, occipital lobe, temporal lobe, paracentral gyrus of the cerebral cortex, pons. Column 2 (A–H), Blank, right cerebellum, corpus callosum, amygdala, caudate nucleus, hippocampus, medulla oblongata, putamen. Column 3 (A–E), Substantia nigra, nucleus accumbens, thalamus, pituitary gland, spinal cord; F–H, blank. Column 4 (A–H), Heart, aorta, left atrium, right atrium, left ventricle, right ventricle, interventricular septum, apex of heart. Column 5 (A–H), Esophagus, stomach, duodenum, jejunum, ileum, ileocecum, appendix, ascending colon. Column 6 (A–C), Transverse colon, descending colon, rectum; D–H, blank. Column 7 (A–H), kidney, skeletal muscle, spleen, thymus, peripheral blood leukocyte, lymph node, bone marrow, trachea. Column 8 (A–H), Lung, placenta, bladder, uterus, prostate, testis, ovary, blank. Column 9 (A–F), Liver, pancreas, adrenal gland, thyroid gland, salivary gland, mammary gland; G and H, blank. Column 10 (A–H), Promyelocytic leukemia (HL-60), HeLa S3, chronic myelogenous leukemia (K-562), lymphoblastic leukemia (MOLT-4), Burkitt's lymphoma (Raji), Burkitt's lymphoma (Daudi), colorectal adenocarcinoma (SW480), lung carcinoma (A549). Column 11 (A–H), Fetal brain, fetal heart, fetal kidney, fetal liver, fetal spleen, fetal thymus, fetal lung, blank. Negative controls: Column 12 (A–H), Yeast total RNA, yeast tRNA, *E. coli* rRNA, *E. coli* DNA, poly r(A), human C_ot-1 DNA, 100 ng human DNA, 500 ng human DNA.

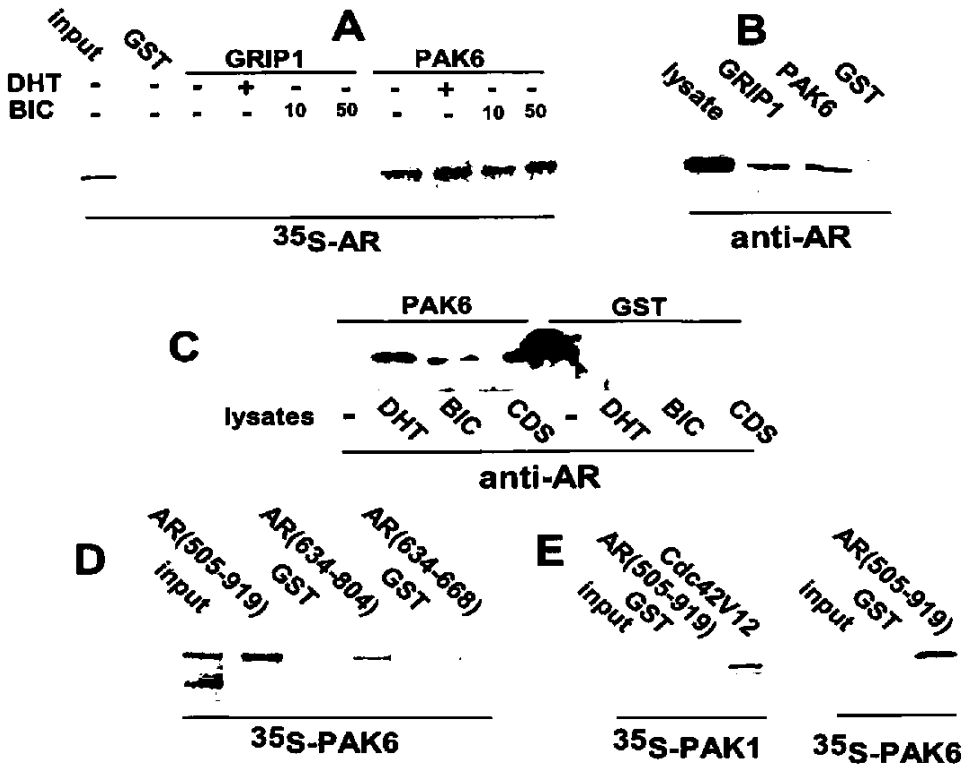


Fig. 4. PAK6 Binds to the AR

A, GST, GST-GRIP1(624–1122), or GST-PAK6(256–681) were used to pull down ^{35}S-labeled AR, plus or minus DHT (10 nM) or BIC (10 or 50 μM), as indicated. B, Lysates from LNCaP cells grown in medium with 10% FCS were precipitated with GST, GST-GRIP1(624–1122), or GST-PAK6(256–681), and bound AR was detected by immunoblotting. C, LNCaP cells grown in medium with 10% CDS FCS or in this medium with 10 nM DHT or 5 μM BIC added overnight were lysed in glycerol lysis buffer, precipitated with GST-PAK6(256–681) or GST beads (25 μg each), and AR immunoblotted. D, ^{35}S-Labeled full-length PAK6 was precipitated with the indicated GST-AR fusion proteins. E, ^{35}S-labeled full-length PAK1 or PAK6 was precipitated with GST, GST-AR(505–919), or GTP-loaded GST-Cdc42V12. The input lanes contain 10% of the material incubated with the beads.

sion in testis and in many areas of the brain, particularly cortical structures (Fig. 2I). Lower level expression was seen in prostate, thyroid, adrenals, placenta, kidney, esophagus, mammary gland, and heart. There was little or no detectable expression in ovary, uterus, intestine, liver, lung, spleen, thymus, peripheral blood leukocytes, lymph node, or bone marrow.

PAK6 Binding to the AR in Mammalian Cells and *in Vitro*

PAK6 binding to the AR in mammalian cells was assessed by fusing the VP16 transactivation domain to the N-terminal of the PAK6 fragment isolated from the yeast screen, VP16-PAK6(256–681). DHT induced AR transcription 60-fold from a luciferase reporter gene regulated by a multimerized androgen response element (ARE_4) (Fig. 3). Cotransfection with vectors encoding only the VP16 activation domain (AASVVP16) or a VP16-SRC1(595–780) fusion, containing the first LXXLL motif of SRC-1, were used as negative controls as this region of SRC-1 was shown previously to interact very weakly or not at all with the AR (10, 11, 16). Both vectors markedly repressed AR activity (Fig. 3 and data not shown), likely reflecting competition for transcription factors by the noninteracting VP16 transactivation domain. In contrast, cotransfection with the VP16-PAK6(256–681) vector increased the induction to 175-fold. These results indicated that PAK6 interacted with the intact ligand-bound AR on DNA in mammalian cells.

AR binding to PAK6 was next assessed *in vitro* by precipitation with a series of glutathione-*S*-transferase (GST) fusion proteins. The fragment of PAK6 isolated in the yeast screen was expressed as a GST fusion protein, GST-PAK6(256–681), and full-length ^{35}S-labeled AR was generated by coupled *in vitro* transcription/translation. AR binding to GST-PAK6(256–681) was compared with binding to a GST-GRIP1 (624–1122) fusion protein, containing multiple LXXLL motifs and shown to interact with agonist-bound LBDs of nuclear receptors including the AR (48, 57). AR binding to the GST-GRIP1(624–1122) fusion protein was detectable, but was very weak (Fig. 4A). Binding was not enhanced by DHT, which may reflect nonnative folding of the *in vitro*-generated AR. In contrast, full-length AR was precipitated efficiently by the GST-PAK6(256–681) fusion protein. The binding was ligand

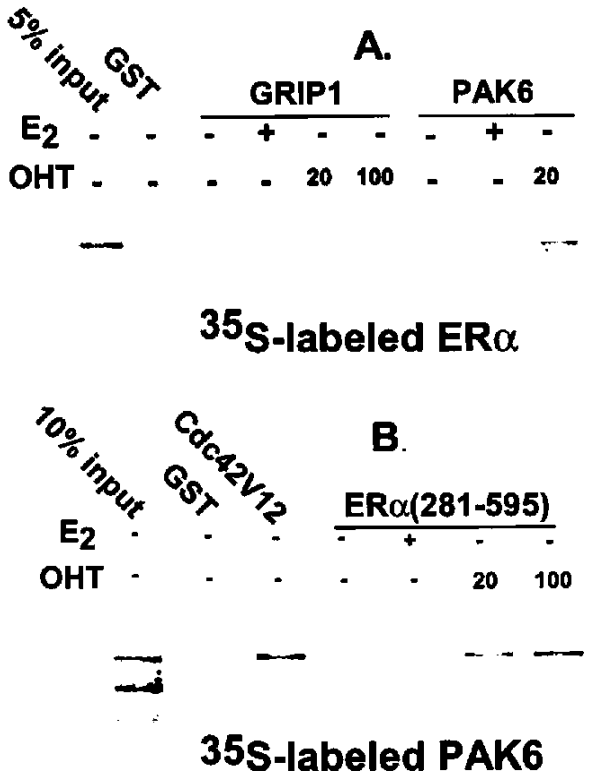


Fig. 5. PAK6 Binds to the ERα

A, The indicated GST fusion proteins were used to pull down ^{35}S-labeled ERα in the presence of 10 nM E2 (E_2), 20 μM or 100 μM OHT, or no added hormone. B, GST, GST-GTP-Cdc42V12, or GST-ERα(281–595) were used to pull down full-length ^{35}S-labeled PAK6, in the presence or absence of the indicated hormones.

independent as it did not require added DHT and was not blocked by bicalutamide (BIC), a competitive antagonist of DHT binding and AR function (58).

GST-PAK6(256–681) pull-down experiments were next carried out using the endogenous AR from LNCaP cells, the only generally available AR-expressing human prostate cancer cell line. LNCaP cells express a mutant AR (T877A) that still responds to DHT but has altered responses to other ligands and AR antagonists (59). LNCaP lysates were precipitated by GST-PAK6(256–681), GST-GRIP1(624–1122), or control GST beads, and AR was detected by immunoblotting. The results similarly demonstrated specific binding of the intact AR to PAK6 (Fig. 4B). In these experiments comparable binding to the GST-GRIP1(624–1122) control was observed, possibly reflecting native folding and androgen binding by the AR *in vivo*. The hormone dependence of binding by the LNCaP AR was further assessed by culturing the cells overnight in medium with charcoal dextran-stripped (CDS) FCS (steroid hormone-depleted medium), or with added DHT (10 nM) or BIC (5 μM), which is also an antagonist of the LNCaP AR. GST-PAK6(256–681) bound specifically to the AR from DHT, BIC, and untreated (CDS) LNCaP cells (Fig. 4C), further demonstrating that binding was not ligand dependent.

Binding of full-length PAK6 to AR was investigated using ^{35}S-PAK6, which was labeled by coupled *in vitro* transcription/translation. Strong binding was observed to a GST-AR(505–919) fusion protein, containing the DBD, hinge, and LBD (Fig. 4D). Binding was also detected to deletion mutants GST-AR(634–804), with the LXXLL binding AF-2 removed, and to GST-AR(634–668). The GST-AR(634–668) protein corresponds to a fragment from the AR nuclear localization signal (amino acids 617–633) to the beginning of helix 3, which marks the beginning of the LBD. These latter results indicated that PAK6 bound to a site distinct from the LXXLL motif binding AF-2 and suggested that binding was through the hinge region between the DBD and LBD.

Finally, the specificity of the PAK6-AR interaction was assessed by examining GST-AR binding to PAK1. In contrast to the results with PAK6, there was no specific binding of *in vitro*-transcribed/translated PAK1 to the GST-AR (505–919) fusion protein (Fig. 4E). However, PAK1 was found to bind to a GTP-loaded GST-Cdc42 fusion protein (see below). This result indicated that the AR interaction was not a general property of PAKs.

PAK6 Binding to ERα

The ERα was next examined to determine whether PAK6 binding was specific for the AR. ^{35}S-Labeled ERα, generated by *in vitro* transcription/translation in rabbit reticulocyte lysates, bound specifically to the positive control GST-GRIP(624–1122) fusion protein (Fig. 5A). This GRIP1 binding in the absence of added estrogen likely reflected estrogen in the rabbit reticulocyte lysate and could be augmented with added E2. As expected, ERα binding to GST-GRIP1(624–1122) was markedly reduced by the partial agonist 4-hydroxytamoxifen (OHT). A comparable level of ERα binding to GST-PAK6(256–681) was observed in the absence or presence of E2. However, in marked contrast to GST-GRIP1 results, ERα binding to PAK6 was enhanced (3.4-fold) by OHT (Fig. 5A). This latter finding was consistent with the AR results and indicated that PAK6 bound to a site distinct from the agonist-generated LXXLL coactivator binding site, as this site is occluded in the OHT-bound ERα (60–62).

It was next determined whether full-length PAK6 bound specifically to the ERα. These experiments used a GST-ERα(281–595) fusion protein, encompassing the region C terminal to the NLS and the complete ERα LBD, and shown previously to bind LXXLL containing coactivator proteins in an agonist-dependent manner (63). Full-length *in vitro*-transcribed/translated PAK6 bound specifically to the GST-ERα(281–595) fusion protein in the absence of added E2, and binding was not augmented by added E2 (Fig. 5B). Moreover, consistent with the results above, binding was enhanced (2.7-fold) by OHT. PAK6 binding by the OHT-ligated GST-ERα was comparable to

the CRIB domain-mediated binding of GST-GTP-Cdc42V12 (see below).

PAK6 Interaction with p21 GTPases

It was next determined whether PAK6 had a functional CRIB domain capable of binding to GTP-Cdc42 and/or -Rac. These experiments used a full-length PAK6 cDNA with a C-terminal myc/his epitope tag, constructed in the pcDNA3 mammalian expression vector. GST pull-down experiments were carried out using GTPase-deficient (activated) Cdc42V12 and RacV12 mutants expressed as GST fusion proteins. Equal amounts of GST fusion proteins bound to glutathione agarose beads were first loaded with GTP. Comparable GTP loading was confirmed on parallel samples using ^{32}P-GTP (not shown). The beads were then used to precipitate the myc/his-tagged PAK6 from transfected CV1 cell lysates. GST-GTP-Cdc42V12 pulled down a substantial amount of PAK6, identified as a 75-kDa protein by immunoblotting with an anti-myc monoclonal antibody (mAb) (Fig. 6A). In contrast, only very weak binding to GST-GTP-RacV12 was detected, although this binding appeared to be specific as no binding was detected to either an inactive control (GST-RacN17) or to other control GST proteins.

GTP-Cdc42 or -Rac binding activates the kinase activity of other PAKs by blocking an autoinhibitory domain carboxy to the CRIB domain (Fig. 1B) (52, 64–66). However, this domain appears to be absent in PAK4–6 (see Fig. 1B), and PAK4 kinase activity is not stimulated by Cdc42 (37). Therefore, kinase assays were carried out to assess PAK6 activation by GTP-Cdc42. For these experiments, full-length ^{35}S-labeled PAK6 was expressed *in vitro* and precipitated by a series of GST fusion proteins bound to glutathione beads. The kinase activity of the precipitated proteins was compared with proteins precipitated by the same beads from a control unprogrammed rabbit reticulocyte lysate, using myelin basic protein (MBP) as a substrate. No kinase activity was detected in the GST-GTP-Cdc42V12 precipitate (Fig. 6B, *top panel*), although PAK6 binding to these beads was confirmed by recovery of the labeled PAK6 protein (Fig. 6B, *bottom panel*). There was also no detectable kinase activity or PAK6 binding to GST-Rac fusion proteins.

Kinase activity was precipitated by the GST-AR(505–919) fusion protein from the unprogrammed and PAK6 programmed lysates (Fig. 6B). However, the kinase activity from the PAK6 programmed lysate was consistently greater (~3-fold). This indicated that it reflected PAK6 kinase activity, or possibly PAK6 activation of another AR- associated kinase, stimulated by AR binding. It is not yet clear whether the lower levels of kinase activity precipitated from the unprogrammed lysate were due to an endogenous PAK or other kinases. Taken together, these results demonstrated that the PAK6 CRIB domain was functional with respect to p21 binding, with a marked preference for binding GTP-Cdc42 *vs.* GTP-Rac. The results further indicated that PAK6 kinase activity was not regulated by Cdc42 binding, with the data suggesting instead a role for AR in regulating PAK6 kinase activity.

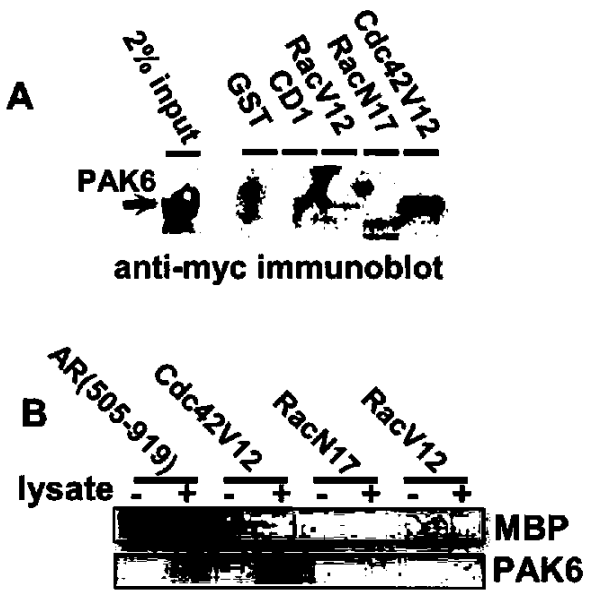


Fig. 6. PAK6 Binding by p21 GTPase and Kinase Activation

A, GST fusion proteins were used to pull down myc-tagged PAK6 from a transfected CV1 cell lysate. Equivalent amounts of GST fusion proteins loaded with GTP were used as indicated, and precipitated PAK6 was detected by immunoblotting with an anti-myc mAb. GST-CD1d is an irrelevant control fusion protein. Lysate alone (2% of the material used for each pull down) is shown in the first lane, and the position of the full-length PAK6 (75 kDa) is indicated. B, PAK6 programmed or unprogrammed rabbit reticulocyte lysates (PAK6 +/−, respectively) were pulled down with the indicated AR or GTP-loaded GST fusion proteins. The precipitates were then split in half and analyzed for kinase activity with MBP substrate (*top panel*), or analyzed for bound ^{35}S-PAK6 (*bottom panel*).

PAK6 Inhibition of AR and ERα Transcriptional Activity

Cotransfection experiments were carried out to determine whether PAK6 could modulate the transcriptional activity of the AR. Cells were cotransfected with an ARE_4-luciferase reporter plasmid, AR expression vector ($pSVAR_o$) (67), pcDNA-PAK6 or control (pcDNA-LacZ) expression vectors, and an internal control Renilla vector (pRL-SV40). AR transcriptional activity was stimulated 33-fold by DHT in the absence of PAK6 and was not inhibited by the control pcDNA-LacZ vector (Fig. 7A). In contrast, AR transcriptional activity was markedly inhibited by PAK6, with induction reduced approximately 5-fold by 200 ng of the PAK6 vector. This inhibition was not a nonspecific effect on transcription or on the pSV promoter regulating the AR, as expression of the control Renilla reporter regulated by a pSV promoter was unaffected by PAK6 (Fig. 7B). Immunoblotting for AR protein further showed that the

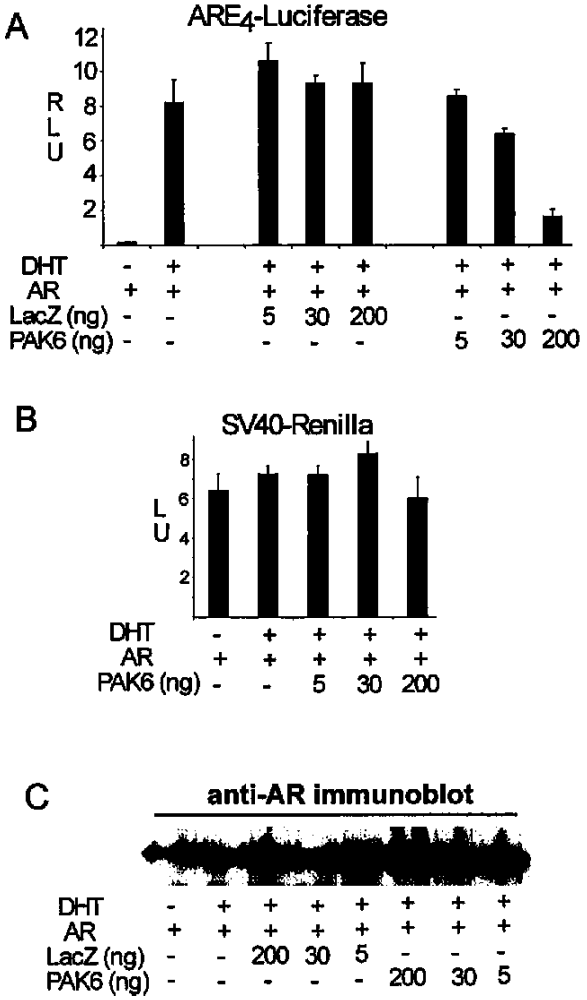


Fig. 7. PAK6 Inhibition of AR Transcriptional Activity

A, CV1 cells were transiently transfected with an ARE_4-luciferase reporter (100 ng), pRL-SV40 (0.2 ng), $pSVAR_o$ (200 ng), and pcDNA-LacZ or pcDNA-PAK6 expression vectors. Luciferase and Renilla activity were assessed after 24 h ± DHT. B, Renilla activity from the experiment in panel A. C, AR expression in lysates from the cells used in panel A.

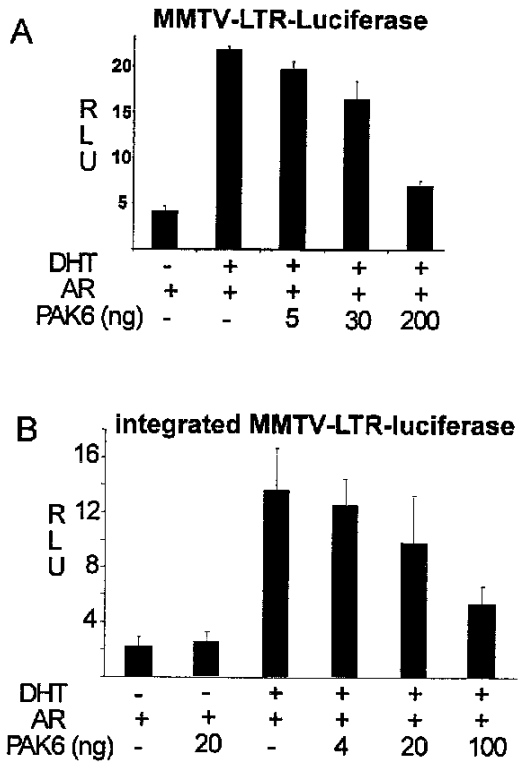


Fig. 8. PAK6 Inhibition of AR on Episomal and Integrated MMTV-LTR

A, CV1 cells were transiently transfected with an MMTV-LTR-luciferase reporter. Cotransfected plasmids and DHT treatments were as in Fig. 7. B, CV1(MMTV-Luc) cells were cotransfected with AR, Renilla, and PAK6 plasmids and treated with DHT as in panel A.

inhibition was not due to decreased AR protein expression (Fig. 7C).

The effect of PAK6 on transcription from another AR-responsive promoter, the mouse mammary tumor virus long terminal repeat (MMTV-LTR) containing two steroid response elements recognized by the AR, was next examined. DHT stimulated AR transcriptional activity on the MMTV-LTR-luciferase reporter by 5.3-fold (Fig. 8A). Similar to the above result with the ARE_4 reporter, this DHT-stimulated activity was inhibited by cotransfection with PAK6. It was also determined whether PAK6 affected AR transcriptional activity in the more physiological setting of an integrated ARE. For this experiment, CV1 cells were stably transfected with the MMTV-LTR-luciferase reporter plasmid, in conjunction with a neomycin resistance plasmid. G418-resistant clones were then screened for ligand-dependent stimulation of luciferase activity by transfected AR, and a clone with a low level of background luciferase activity and a relatively high level of DHT-inducible AR activity was selected. DHT augmented the transcriptional activity of the transfected AR by approximately 6-fold in these cells (Fig. 8B). This was reduced to 2.4-fold induction by cotransfection with PAK6, indicating that PAK6 could inhibit AR activity on an integrated as well as an episomal reporter.

Similar cotransfection experiments were carried out with an ERα expression vector and an estrogen responsive element (ERE)-regulated luciferase reporter plasmid to determine whether PAK6 modulated ERα transcriptional activity. Luciferase activity was stimulated about 4.5-fold by E2 (Fig. 9A). Cotransfection with PAK6 reduced this stimulation by approximately 50%, while a control LacZ vector had no effect. This result demonstrated a similar inhibitory effect of PAK6 on AR and ERα transcriptional activity.

As PAK6 also binds to GTP-Cdc42, it was possible that AR and ERα inhibition by PAK6 was due to sequestration of GTP-Cdc42 rather than a direct interaction. This was addressed by cotransfection with activated (GTPase-deficient) Cdc42V12. DHT stimulated AR transactivation by 26-fold, and this was re-

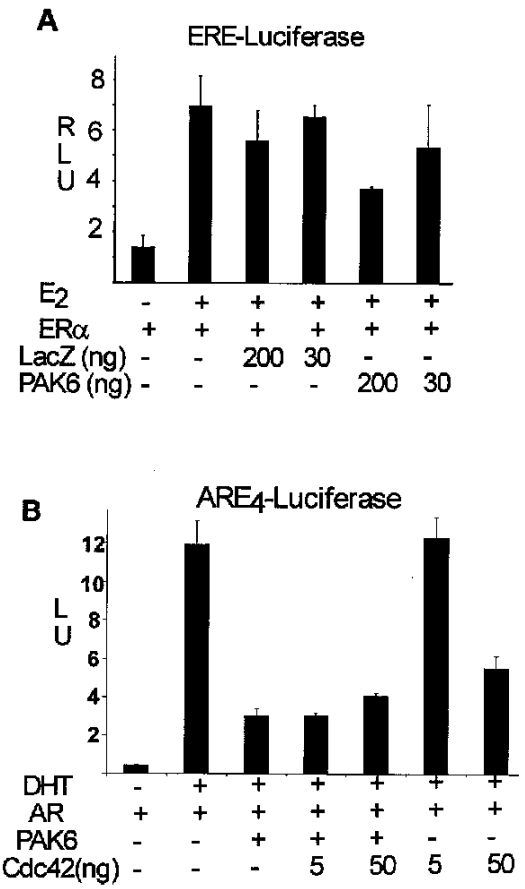


Fig. 9. PAK6 Inhibition of ERα and Effect of Cdc42

A, CV1 cells were transiently transfected with an ERE_2-luciferase reporter (100 ng), pRL-SV40 (0.2 ng), pcDNA-ERα (200 ng), and pcDNA-LacZ or pcDNA-PAK6 expression vectors. Luciferase and Renilla activities were assessed after 24 h ± E2 (10 nM). B, CV1 cells were transfected and treated as in Fig. 7, with the addition of a pCDNA-Cdc42V12 expression plasmid as indicated. Results in this experiment were not corrected for Renilla, as Cdc42V12 at the high concentration decreased Renilla activity.

duced to approximately 7-fold by PAK6 (Fig. 9B). This repression by PAK6 was not reversed by cotransfected Cdc42V12. Moreover, transfection of Cdc42V12 by itself inhibited AR transcriptional activity, consistent with a recent report that Rho GTPases can negatively regulate steroid hormone receptors (68). Therefore, these results demonstrated that AR inhibition by PAK6 was not due to sequestration of Cdc42.

DISCUSSION

This study identified PAK6 as a strongly AR-interacting protein in a yeast two-hybrid screen. The physiological significance of the interaction was supported by *in vitro* studies demonstrating specific binding of the full-length AR and PAK6 proteins and by mammalian one-hybrid experiments demonstrating an interaction between the ligated native AR and a VP16-PAK6 fusion protein. PAK6 also bound to ERα, and this binding was enhanced by OHT, indicating that the interaction was sensitive to the functionally critical conformational changes in the AF-2 region that mediate coactivator binding to the ERα and to other nuclear receptors in response to ligand (61, 62, 69, 70).

The enhanced ERα binding with OHT further indicated that the PAK6 interaction was not through the LXXLL binding AF-2 region, as this site is occluded in the OHT- bound ERα (62). This was consistent with the ligand independence of AR binding *in vitro* and results using GST-AR deletion mutants that indicated a binding site in the hinge region between the DBD and LBD. A number of other proteins appear to bind to this region of the AR and/or other steroid hormone receptors, including ANPK (a nuclear serine/threonine kinase) (71), L7/SPA (72), and UBC9 (a SUMO conjugating enzyme) (73). Although these proteins can function as coactivators, a deletion in the hinge region enhances AR transcriptional activity (74). Moreover, mutations at the C terminus of the AR hinge region have been identified in human prostate cancers and in an SV40 T/t antigen-induced mouse prostate cancer (75), with the latter mutation enhancing AR transcriptional activity, consistent with a corepressor binding to this region.

AR transcriptional activity from two different episomal reporter genes, as well as an integrated reporter, was inhibited by PAK6. Inhibition was not due to Cdc42 sequestration by the PAK6 CRIB domain, as it was not reversed by cotransfected Cdc42V12. Moreover, Cdc42V12 by itself inhibited AR transcriptional activation. This latter result was consistent with a previous report showing that Rho GDIα, a negative regulator of Rho GTPases, could augment AR, ERα, ERβ, and GR transcriptional activity, and that the ER was inhibited by Rho, Rac, and Cdc42 (68). Taken together, these results suggest that the AR transcriptional inhibition by PAK6 could be due to recruitment of Cdc42 to the AR complex. However, PAK6 is unlikely to mediate all of the effects of Rho GTPases on steroid hormone receptors, as it interacted only weakly with GTP-Rac.

Alternatively, PAK6 inhibition of the AR may be mediated by phosphorylation of the AR or other AR-associated proteins. Studies addressing possible substrates for PAK6 kinase activity are underway but have not found AR phosphorylation by PAK6 *in vitro* or increased AR phosphorylation *in vivo* in response to transfected PAK6. Other possible mechanisms for AR inhibition by PAK6 are that PAK6 competes with coactivators for binding, stabilizes the AR in a conformation unfavorable for coactivator binding, or blocks the interaction between the AR N-terminal domain and LBD (12–18). These latter mechanisms would be consistent with the enhanced PAK6 binding to the OHT- ligated ERα, as OHT blocks coactivator binding. Finally, it should of course be emphasized that AR

inhibition may reflect nonphysiological high levels of transfected PAK6, and that PAK6 may instead selectively modulate AR activity on particular promoters or in response to activation of other signal transduction pathways.

Alternative functions for the PAK6-AR interaction may be to recruit PAK6 and/or activate its kinase activity. The kinase activity of most previously characterized PAKs is blocked by an autoinhibitory domain that follows the CRIB domain (52, 64–66). Cdc42 or Rac binding relieves this inhibition and results in PAK autophosphorylation and activation of kinase activity. Although PAK6 clearly has a functional CRIB domain, which selectively binds to Cdc42, there is limited homology between PAK6 and human PAK1–3 in the CRIB-regulated autoinhibitory domain (see Fig. 1B), and PAK6 kinase activity is not activated by GTP-Cdc42 binding. The N terminus of PAK6 is homologous to PAK4, which also selectively binds to Cdc42 and is not activated by Cdc42 binding (37). Studies are underway to determine whether, and under what conditions, the AR can activate PAK4 or PAK6 kinase activity *in vivo*, as suggested by the *in vitro* kinase activity associated with PAK6 bound to a GST-AR fusion protein. This kinase activity could contribute to the rapid nontranscriptional activation of MAPKs and other pathways demonstrated previously for ERα (19, 20) and AR (21).

The cellular distribution of transfected PAK6, plus or minus cotransfected AR, was primarily in the cytoplasm and on plasma membrane. However, lower levels of nuclear PAK6, alone or in association with AR, could not be ruled out. Preliminary biochemical fractionation studies similarly indicate that PAK6 is mostly cytoplasmic but suggest that a small fraction might be nuclear. The highest levels of PAK6 expression were in brain and testis, although PAK6 could also be expressed at relatively high levels by specific cell types in other tissues. While this manuscript was under revision, another group similarly identified PAK6 as an AR-interacting protein that was highly expressed in testis and could repress AR transcriptional activity, although their data indicated marked AR stimulated nuclear translocation of transfected PAK6 (76). It is clear that specific antibodies will be needed to better assess the cellular and tissue distribution of the endogenous protein.

Database searches have not revealed definite homologs of PAK6 in other species. However, the mushroom bodies tiny *(mbt)* gene from *Drosophila* encodes a PAK that appears related to human PAK4, 5, and 6 (77). Mutations in *mbt* interfere with brain development, which in conjunction with the high level expression of PAK6 in human adult and fetal brain, suggest a role for PAK6 in brain development. In this regard, a PAK3 mutation has been identified in a family with mental retardation (51). However, although the biological functions of PAK6 remain unclear, they likely differ in detail from most previously described PAKs based upon low-sequence homology, the absence of SH3 binding motifs that direct binding of the Nck adapter protein (31, 53–55) or the Pix/Cool nucleotide exchange proteins (78, 79), and the failure of GTP-Cdc42 to activate PAK6 kinase activity. PAK6 binding may be a mechanism through which diverse signal transduction pathways, in particular those mediated through Cdc42, can modulate the activity of the AR and ERα (and possibly other nuclear receptors). Alternatively, or in addition, PAK6 binding may mediate nontranscriptional functions of the AR or ERα. Finally, while the OHT-enhanced PAK6-ERα interaction may or may not be physiological, it could contribute to the therapeutic effects of OHT in breast cancer and in other tissues.

MATERIALS AND METHODS

PAK6 Cloning

A fragment of the human AR from glycine 505 to the C terminus (AR505–919) was generated by PCR and cloned into the pAS2 yeast GAL4 DNA binding domain vector (CLONTECH Laboratories, Inc., Palo Alto, CA). A series of human GAL4 activation domain libraries were screened in the presence of 1 μM DHT, and a fragment of PAK6 was isolated from a prostate library in the pACT2 vector (clone 56). A plasmid containing the N terminus of PAK6, identified as an EST from a testis library (GenBank accession no. AA815255), was obtained from the I.M.A.G.E. (Integrated Molecular Analysis of Genomes and their Expression) consortium. Additional AR yeast vectors, as indicated, were constructed by PCR and confirmed by DNA sequencing. pGAD24-GRIP(563–1121) was from Michael Stallcup (University of Southern California, Los Angeles) (57). Liquid β-galactosidase assays were carried out on extracts from transformed yeast containing an integrated GAL4 promoter regulating β-galactosidase (strain HF7c) (CLONTECH Laboratories, Inc.) with *O*-nitrophenyl-β-D-galactopyranoside as the substrate, as described by the manufacturer.

PAK6 Expression Vectors

Full-length PAK6 was assembled from three fragments in the pcDNA3.1(−)/Myc-His C mammalian expression vector, containing C-terminal myc and histidine epitope tags (Invitrogen, Carlsbad, CA). The 5′-end through an internal *Xba*I site was obtained from the I.M.A.G.E. consortium plasmid described above, a middle fragment from the *Xba*I site to a downstream *Bam*HI site was generated by PCR amplification from prostate cancer cDNA, and a fragment from the *Bam*HI site to the 3′-end was from the yeast clone 56. The pcDNA 3.1 epitope tag (myc-his) was placed in frame at the C terminus by cutting at a *Blp*I site in the PAK6 stop codon and a downstream *Kpn*I site in the pcDNA polylinker, followed by blunting with mung bean nuclease and ligation.

The GFP-PAK6 vector (pEGFP-PAK6) was constructed in the pEGFP-C1 vector (CLONTECH Laboratories, Inc.). An oligonucleotide encoding the first 11 amino acids of PAK6 with a C-terminal *Bgl*II site was ligated to the *Bgl*II site encoding amino acids 11 and 12 in PAK6. This was then excised and an N-terminal *Hin*dIII site introduced by the oligonucleotide was used to ligate PAK6 in frame at the C terminus of GFP as a *Hin*dIII-*Kpn*I fragment. A GFP-AR expression vector (pEGFP-AR) was similarly constructed in pEGFP-C1, but using an *Eag*I site located eight amino acids from the N terminus of the AR. To generate GST-PAK6 (256–681), an *Xho*I site was introduced into the pGEX-2TK polylinker, and

the entire PAK6 fragment from clone 56 was inserted in frame using a compatible *Sal*I site in pACT2 at the junction between the GAL4 activation domain and PAK6.

PAK6 Expression

pEGFP-PAK6, with or without $pSVAR_o$, was transfected into HeLa cells by electroporation. Cells were then plated onto coverslips, cultured for 24 h in DMEM with 10% charcoal-dextran stripped FCS (HyClone Laboratories, Inc., Logan, UT), treated for varying times with DHT, and fixed in 1% paraformaldehyde in PBS. HeLa cells were similarly transfected with pEGFP-AR, with or without the pcDNA-PAK6 expression vector. A human multitissue blot containing polyadenylated RNA from multiple different human tissues and cell lines (Human Multiple Tissue Expression Array, CLONTECH Laboratories, Inc.) was probed with a PCR-generated ^{32}P-labeled fragment of PAK6 corresponding to amino acids 115–383 (having no homology to previously described PAKs or other proteins). Hybridization and washing conditions were as recommended by the manufacturer.

GST Fusion Proteins and Pull Downs

GST-AR fusion proteins were constructed in pGEX-2TK by PCR amplification and confirmed by sequencing (80). GST-GRIP1(624–1122) and GST-ER(281–595) (63) were from Myles Brown (Dana Farber Cancer Institute, Boston, MA). GST-RacV12, -RacN17, and -Cdc42V12 mutant constructs were from Chris Carpenter (Beth Israel Deaconess Medical Center, Boston, MA) (81). For GTP loading, the GST-RacV12 and GST-Cdc42V12 fusion proteins bound to glutathione agarose beads (5 μg) were initially incubated in 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT), with a 10-fold molar excess of GTPγS, for 15 min at 30 C. The beads were then placed on ice, and $MgCl_2$ was added to a concentration of 5 mM. After 5 min on ice, the beads were pelleted and lysates were added. GTP loading was comparable for the Rac and Cdc42 fusion proteins, based upon [γ-^{32}P]GTP binding in parallel experiments.

For GST pull-down experiments, CV1 cells were transfected in 10-cm plates with 10 μg of PAK6 in pcDNA3.1(−)/Myc-His C, using Lipofectamine according to the manufacturer's directions (Life Technologies, Inc., Gaithersburg, MD). Transfected CV1 cells or LNCaP prostate carcinoma cells (with endogenous AR) were lysed in 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 5 mM DTT, and protease inhibitors for 15 min at 4 C, followed by centrifugation to remove nuclei. Lysates were then incubated with GST fusion proteins (5 μg except where indicated) bound to glutathione agarose beads for 2–4 h at 4 C, followed by washes in lysis buffer and elution in SDS-PAGE sample buffer. In the indicated experiments lysis and washes were in 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 10% glycerol, 0.1% Triton X-100, and protease inhibitors (glycerol lysis buffer). PAK6 was detected by immunoblotting with mouse anti-myc mAb 9E10, and AR was detected with a mixture of rabbit anti-AR antibodies against N and C-terminal peptides (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

^{35}S-labeled proteins were generated by *in vitro* transcription/translation (TNT T7 Quick Coupled Transcription/Translation System, Promega Corp., Madison, WI). AR, ERα (from Myles Brown, Dana Farber Cancer Institute), PAK1, and PAK6 plasmids (in pGEM3 or pcDNA, 2 μg) in 50 μl of reticulocyte lysate with 20 μCi of ^{35}S-methionine were incubated at 30 C for 1 h, according to the manufacturer's directions. GST fusion proteins (5 μg on glutathione agarose beads) were mixed with 10 μl of the programmed lysate in 0.5 ml of PBS, pH 7.4, 1 mM DTT, and protease inhibitors. After 2–4 h at 4 C, the beads were washed in the same buffer, except with 0.05% NP-40. Proteins were eluted in SDS-PAGE sample buffer, and labeled proteins were detected with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). DHT was obtained from Sigma, OHT was from Alexis Biochemicals, and bicalutamide was kindly provided by Astra Zeneca Pharmaceuticals (Wilmington, DE).

Kinase Assays

Beads with precipitated proteins were washed once in kinase buffer (40 mM HEPES, pH 7.4, 20 mM $MgCl_2$) and then resuspended in 30 μl of kinase buffer with MBP (5 μg) (Sigma), 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and 20 μM cold ATP. Reactions were carried out at room temperature for 15 min and stopped with SDS-PAGE loading buffer containing 10 mM EDTA. Labeled MBP was analyzed on 15% SDS-PAGE.

AR and ERα Transcriptional Activity

Transient transfections to assess AR transcriptional activity were carried out using CV1 cells in 24-well plates (58, 82). Cells were transfected using LipofectAMINE or LipofectAMINE 2000 (Life Technologies, Inc.) with AR expression vector ($pSVAR_o$) (67), a Renilla expression vector to control for transfection efficiency (pRL-CMV or pRL-SV40, Promega Corp.), and PAK6 or other experimental or control plasmids as indicated. The VP16-SRC(595–780) encodes the first nuclear receptor binding domain of SRC-1 fused to the C terminus of the VP16 activation domain in the AASVVP16 vector (83). A VP16-PAK6(256–681) expression vector was generated in AASVVP16 by PCR to generate an in-frame *Eco*RI site at amino acid 256 in PAK6 and a *Hin*dIII site at the 3′-end. The fragment was then cloned at the 3′-end of VP16 as an *Eco*RI-*Hin*dIII fragment. The reporter plasmids were an androgen-responsive luciferase reporter construct driven by synthetic AREs and a minimal promoter (ARE_4-luciferase) or MMTVpA3Luc, driven by the androgen-responsive MMTV-LTR (83). The ARE_4-luciferase reporter was constructed by inserting four tandem ARE repeats (5′-TGTACAGGATGT-TCTGAATTCCATGTACAGGATGTTCT-3′) in front of an E1b minimal TATA box sequence, followed by a firefly luciferase gene. After the 24-h transfection, cells were cultured for another 24 h in DMEM with 5% CDS FCS, with or without added 10 nM DHT. Lysates were assayed for luciferase activity and Renilla activity using a dual luciferase kit (Promega Corp.), and luciferase activity was normalized for Renilla to give relative light units.

ERα transcriptional activity was assessed similarly using a pcDNA3.1-ERα expression vector and a luciferase reporter gene, ERE_2TK-Luc, regulated by two copies of the ERE from the vitellogenin gene (kindly provided by Myles Brown, Dana Farber Cancer Institute). The ERα experiments were done in phenol red-free medium. All points were in triplicate or quadruplicate and mean ± SEM are shown.

Additional experiments used CV1 cells containing an integrated luciferase reporter gene regulated by the androgen responsive MMTV-LTR. These CV1(MMTV-Luc) cells were generated by transfecting CV1 cells with an MMTV-LTR-luciferase reporter plasmid (MMTVpA3Luc) (83) and neomycin resistance plasmid, and selecting for G418-resistant cells. A series of clones were then screened to identify ones with low background luciferase activity and high level androgen-dependent AR induction.

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